

***Ophiostoma* species, including *Ophiostoma borealis* sp. nov., infecting wounds of native broad-leaved trees in Norway**

Gilbert KAMGAN NKUEKAM^a, Halvor SOLHEIM^b, Z. Wilhelm DE BEER^a,
Joha W. GROBBELAAR^c, Karin JACOBS^d,
Michael J. WINGFIELD^a & Jolanda ROUX^a

^aDepartment of Microbiology and Plant Pathology,

^cDepartment of Genetics, Forestry and Agricultural Biotechnology Institute (FABI),
University of Pretoria, Pretoria, South Africa,
email: gilbert.kamgan@fabi.up.ac.za

^bNorwegian Forest and Landscape Institute, P. O. Box 115, N-1431 Ås, Norway

^dDepartment of Microbiology, University of Stellenbosch, Stellenbosch,
South Africa

Abstract – *Ophiostoma* spp. include important pathogens of trees and causal agents of sapstain. These fungi infect wounds on trees and are typically carried by insects, especially bark beetles. *Ophiostoma* spp. on coniferous hosts in the Northern Hemisphere are well-known. However, other than for the serious pathogens *O. ulmi* and *O. novo-ulmi*, very little research has been done on the occurrence of this group of fungi on native broad-leaved trees, especially in the Nordic countries. In this study, surveys were conducted in several areas of Norway to isolate *Ophiostoma* spp. associated with wounds on native broad-leaved trees belonging to the genera *Betula*, *Fagus*, *Quercus*, *Sorbus* and *Tilia*. Morphological studies and comparisons of DNA sequences for the ITS, 5.8S and part of the beta-tubulin gene regions were used to confirm the identity of the fungi collected. *Ophiostoma* spp., and especially their *Pesotum* anamorphs, were common on wounds on the trees sampled. In most cases, they were associated with wood stain. *Ophiostoma* spp. collected included predominantly *O. quercus*, *O. borealis* sp. nov., and *O. denticiliatum*. The results of this study emphasize that the diversity of *Ophiostoma* spp. on broad-leaved trees is still incompletely understood in Norway and other European countries.

Forestry / Hardwood trees / Ophiostomatoid / Ophiostomatales / Sapstain

INTRODUCTION

The Ophiostomatales (Ascomycetes) include many important pathogens and causal agents of sapstain in lumber. Amongst the pathogens, the Dutch elm disease fungi, *O. ulmi* (Buisman) Nannf. and *O. novo-ulmi* Brasier, are by far the best known (Wingfield *et al.*, 1993; Brasier, 2000). Most species of *Ophiostoma* Sydow & P. Sydow, however, cause sapstain of freshly exposed wood and they can reduce the commercial value of wood products substantially (Wingfield *et al.*, 1993).

The Ophiostomatales accommodate the genera *Ophiostoma* (with *Pesotum* J.L. Crane & Schokn. and *Sporothrix* Hektoen & C.F. Perkins anamorphs), *Ceratocystiopsis* H.P. Upadhyay & W.B. Kendr. (with *Hyalorhinocladiella* H.P. Upadhyay & W.B. Kendr. anamorphs), and *Grosmannia* Goid. (with *Leptographium* Lagerb. & Melin anamorphs) (Zipfel *et al.*, 2006). In their sexual form, these fungi typically produce long-necked ascomata with sticky spores at their apices, which facilitate insect dispersal. Similarly, with the exception of *Sporothrix* spp., most of the asexual structures are adapted for insect dispersal, producing their conidia in sticky droplets on erect conidiophores such as species of *Hyalorhinocladiella*, *Pesotum* and *Leptographium* (Wingfield *et al.*, 1993).

Ophiostoma spp. infect wounds on trees, and are commonly vectored by insects, most often bark beetles (Coleoptera: Scolytinae), with which many of these fungi have specific associations (Six, 2003; Harrington, 2005). However, casual arthropod visitors of wounds such as mites, nitidulid beetles, and flies (Six, 2003; Harrington, 2005) are also important vectors of *Ophiostoma* spp. Gibbs (1980) and Juzwik & French (1983), showed that insects will, under certain circumstances, carry a mixed inoculum of both sapstain and pathogenic fungi. In some cases, the sapstain fungi can out-compete the pathogen and prevent further infection of wounds. This form of mutualism has been demonstrated between sap-feeding beetles, the oak wilt fungus *Ceratocystis fagacearum* (Bretz) J. Hunt and *O. quercus* (Georgev.) Nannf., a well-known sapstain fungus on hardwood trees (Gibbs, 1980; Juzwik *et al.*, 1998).

Many *Ophiostoma* spp. have been reported from Scandinavia. In this regard, the type species of *Ophiostoma*, *O. piliferum* (Fr.) Sydow & P. Sydow, was described from pine and spruce in Sweden as long ago as 1823 (Fries, 1823). Early studies on these fungi in Scandinavia focused mainly on wood and/or pulp staining fungi in Sweden (Lagerberg *et al.*, 1927; Melin & Nannfeldt, 1934) and Norway (Robak, 1932) and more recently in Norway by Roll-Hansen & Roll-Hansen (1980a, b). However, the majority of these studies have dealt with fungal associates of conifer-infesting bark beetles in Sweden (Mathiesen, 1950, 1951; Mathiesen-Käärik, 1953, 1960; Solheim & Långström, 1991), Norway (Solheim, 1986, 1992a, b, 1993) and Finland (Viiri, 1997). Until recently, the only records of *Ophiostoma* spp. on hardwood trees in Scandinavia were of the Dutch elm disease fungus, *O. ulmi*, reported from both Sweden (Mathiesen-Käärik, 1960) and Norway (Roll-Hansen, 1985; Venn, 1986), and of *O. piceae* (Münch) Syd. & P. Syd. from decayed *Betula pubescens* Ehrh. in Norway (Venn, 1972). In two recent studies of the fungal associates of the birch bark beetle, *Scolytus ratzeburgi* Janson, several *Ophiostoma* spp. were isolated from the beetles and their galleries in Finland (Linnakoski *et al.*, 2008). These included the new species, *O. karelicum* Linnakoski, Z.W. de Beer & M.J. Wingf., *O. quercus* and an undescribed species closely related to *O. catonianum* (Goid.) Goid. (Linnakoski *et al.*, 2008). In a subsequent study conducted in Norway, *O. karelicum* was also found to be the most common associate of *S. ratzeburgi* (Linnakoski *et al.*, 2009). In addition, *O. quercus*, together with four unknown species, one of which was described as *O. denticiliatum* Linnakoski, Z.W. de Beer & M.J. Wingf., were found. There were not enough isolates of the remaining species to support the description of new taxa, but they were shown to be closely related to *O. canum* (Münch) Syd. & P. Syd., *O. flexuosum* H. Solheim, and *O. multiannulatum* (Hedgec. & R.W. Davidson) Hendr., respectively (Linnakoski *et al.*, 2009).

The expanding global timber trade poses increasing risks regarding the introduction of alien bark beetles and fungal pathogens into countries worldwide

(Skarpaas & Økland, 2009). Global warming represents another factor that is promoting the invasion of wood and tree-infesting insect pests such as bark beetles into areas previously unsuitable for them to survive (Logan *et al.*, 2003; Raffa *et al.*, 2008). Knowledge regarding the geographic origin and host range of fungal pathogens or fungi that might be commercially relevant is thus important for future pest risk assessments and for the development of effective quarantine and management strategies. Following this objective and as part of a collaborative project between research organizations in Norway and South Africa, funded by the Norwegian and South African Governments, a study was undertaken to survey for and to identify *Ophiostoma* spp. that infect wounds on native broad-leaved tree species in Norway. Specifically the study aimed to address the paucity of detailed knowledge pertaining to this group of potentially important fungi in the country.

MATERIALS AND METHODS

Collection of isolates

Surveys of native broad-leaved trees in Norway were conducted in June and July 2004 and 2005. Sampling focused on wounds artificially induced on trees, freshly cut stumps of *Betula* spp. and *Populus tremula* L., logs and bark flaps of *Betula* spp. (*B. pendula* Roth and *B. pubescens*), *P. tremula*, *Quercus* spp. [*Q. petraea* (Matt.) Liebl. and *Q. robur* L.] and *Salix* spp. log ends at saw mills and loading depots. In 2004, sampling was restricted to forests in eastern Norway mainly around the town of Ås situated in the Boreonemoral vegetation zone, and a loading depot in South Western Sweden, near the town of Filipstad in the Southern Boreal Zone and not far from the Norwegian border. In 2005, sampling was done in both the southernmost part of Norway, in the Nemoral vegetation zone, and in many municipalities in Tromsø county in northern Norway, situated in the Middle Boreal vegetation zone.

For the artificially induced wounds, an axe was used to remove sections of bark ($10 \times 20 \text{ cm}^2$) from living trees growing near lake Årungen in Ås, to expose the cambium. An uneven number of trees were wounded, depending on availability, including nine *Betula* spp., four *Quercus* spp., four *Sorbus aucuparia* L., four *Salix* spp. and one *P. tremula* tree. The wounds were inspected up to two months after they had been made. Pieces of bark and wood including cambial tissue were collected from all wounds that had signs of ophiostomatoid fungal infection (ascomata, synnemata, sap-staining) when examined with a 10X magnification hand lens. Sampling from freshly cut stumps and log ends at saw mills and loading depots was also done in a similar fashion by chopping pieces of infected tissues, especially where bark flaps protecting the wounds from desiccation were visible. Samples were stored in brown paper bags and transported to the laboratory. Dry samples were sprayed with water and sealed in plastic bags to induce sporulation of the fungi. Samples were examined daily for the development of fruiting bodies characteristic of *Ophiostoma* spp. and their anamorphs.

Isolations of the fungi were made by lifting spore masses directly from fruiting structures with a sterile needle onto 2% malt extract agar [MEA: 20 g

malt extract, 15 g agar, Biolab, Midrand, South Africa and 1000 ml deionised water] containing 0.05 g/l of the antibiotic streptomycin (Sigma-Aldrich, Steinheim, Germany). Cultures were grown at 24°C for seven days to obtain pure colonies. Apart from isolates obtained during the survey, additional isolates of *O. quercus* from Austria, Uganda, and South Africa were included in the study for comparative purposes (Tab. 1). Replicates of each isolate were deposited in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa and the culture collection of the Norwegian Forestry and Landscape Institute (NFLI) in Ås. In addition, the ex-type cultures of the new species were deposited at the CBS (Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands).

Morphology of cultures

After purification, isolates were grouped into morphotypes based on culture morphology on MEA. From these morphotypes single drops of conidia or ascospores from selected isolates, or small pieces of mycelium, were transferred to Oatmeal agar media (OMA: 30 g Oats, 20 g Biolab agar and 1000 ml deionised water) to promote sporulation and for comparison with previously published characteristics. Cultures were incubated at 24°C until sporulation and then grouped into morphotypes based on colony colour (Rayner, 1970) and macro-morphology.

Micromorphology

Fifty measurements of all characteristic morphological features were made for isolates chosen as the types for the new species and ten measurements were made for additional isolates. Measurements were recorded as (minimum – mean minus st. dev. – mean plus st. dev. – maximum). The means were then calculated for relevant morphological structures.

DNA isolation, PCR and DNA sequencing

Isolates, representing each of the different morphological groups were selected for DNA sequence comparisons. Single spore drops from pure cultures were transferred to 2% MEA and cultures were grown for 10 days. Mycelium was then scraped from the surface of cultures and transferred to 1.5 ml Eppendorf tubes using a sterile scalpel. DNA was extracted using the protocol described by Möller *et al.* (1992), except that 10 µl of RnaseA (Roche Diagnostics) was added at the final step and incubated overnight at room temperature to digest RNA. The presence of DNA was verified by separating an aliquot of 5 µl on 1% agarose gels, containing ethidium bromide and visualized under Ultraviolet light. The internally transcribed spacer regions (ITS1 & 2) and 5.8S gene of the ribosomal RNA operon were amplified using an Eppendorf Mastercycler (Merck, Hamburg, Germany) and primers ITS1 and ITS4 (White *et al.*, 1990). Parts of the beta-tubulin gene region were also amplified, using primers T10 (O'Donnell & Cigelnik, 1997) and Bt2b (Glass & Donaldson, 1995). The PCR and DNA sequencing protocols described by Kamgan Nkuekam *et al.* (2008a) were followed.

Table 1. *Ophiostoma* isolates obtained in this study from native broad-leaved trees in Norway and Sweden. Also listed are isolates from Finland and Norway from the studies of Linnakoski *et al.* (2008, 2009), as well as isolates from elsewhere sequenced for reference purposes. Accession numbers of sequences obtained in the present study are printed in bold type.

Species	^a CMW no.	ITS		β -tubulin		Hosts	Origin	Collectors
		HT ^b	Acc. no.	HT ^b	Acc. no.			
<i>O. quercus</i>	5928	E	EF408598			<i>Acacia mearnsii</i>	Uganda	Roux
	15528	G	EF408579	10	GQ249305	<i>Betula</i> sp.	Norway, Årungen	Roux, Solheim
	15544	E	EF408581			<i>Sorbus</i> sp.	Norway, Årungen	Roux
	15592	B	GQ249322			<i>Populus</i> sp.	Norway, Årungen	Roux, Solheim
	15608	G	=EF408579	10	=GQ249305	<i>Betula</i> sp.	Sweden, Filipstad	Roux, Solheim
	15704	H	GQ249324			<i>Quercus</i> sp.	Norway, Årungen	Roux
	17828	E	=EF408586	8	GQ249303	<i>Quercus robur</i>	Austria	Kirisits
	17877	E	EF408588			<i>Tilia cordata</i>	Austria	Kirisits
	17892	E	EF408586			<i>Q. robur</i>	Austria	Kirisits
	17893	H	EF408590	8	=GQ249303	<i>Fagus sylvatica</i>	Austria	Kirisits
	17894	E	EF408589			<i>T. cordata</i>	Austria	Kirisits
	19182	G	=EF408579	1	GQ249299	<i>Populus</i> sp.	Norway, Tresnes	Kamgan, Solheim
	19187	F	GQ249323			<i>Quercus</i> sp.	Norway, Øydna	Kamgan, Solheim
	19193	H	=EF408590	16	GQ249310	<i>Populus</i> sp.	Norway, Øydna	Kamgan, Solheim
	19198			8	GQ249302	<i>Quercus</i> sp.	Norway, Øydna	Kamgan, Solheim
	19201			11	GQ249306	<i>Quercus</i> sp.	Norway, Øydna	Kamgan, Solheim
	19214			15	GQ249309	<i>Quercus</i> sp.	Norway, Øydna	Kamgan, Solheim
	19231			7	GQ249301	<i>Quercus</i> sp.	Norway, Salthaug	Kamgan, Solheim
	19240			5	GQ249300	<i>Quercus</i> sp.	Norway, Salthaug	Kamgan, Solheim
	19241			14	GQ249308	<i>Quercus</i> sp.	Norway, Salthaug	Kamgan, Solheim
	19243			9	GQ249304	<i>Quercus</i> sp.	Norway, Salthaug	Kamgan, Solheim
	19257			12	GQ249307	<i>Quercus</i> sp.	Norway, Lyngdal	Kamgan, Solheim
	20452	D	EF408563			<i>Rapanea melanophloeos</i>	South Africa	Roux

^a CMW = Culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.
^b Published sequences of *O. quercus* were assigned to haplotype (HT) groups (numbered alphabetically for ITS and numerically for β -tubulin), each group consisting of identical sequences. Only one sequence per group was deposited in Genbank.
^c Isolates identified as *O. catenianum*-like by Linnakoski *et al.* (2008).
^T Ex-type isolates.

Table 1. *Ophiostoma* isolates obtained in this study from native broad-leaved trees in Norway and Sweden. Also listed are isolates from Finland and Norway from the studies of Linnakoski *et al.* (2008, 2009), as well as isolates from elsewhere sequenced for reference purposes. Accession numbers of sequences obtained in the present study are printed in bold type. (continued)

Species	<i>a</i> CMW no.	ITS		β -tubulin		Hosts	Origin	Collectors
		<i>HT^b</i>	Acc. no.	<i>HT^b</i>	Acc. no.			
<i>O. quercus</i>	29491	A	FJ804488	10	FJ804501	<i>S. ratzeburgi</i> ex <i>Betula</i> sp.	Norway, Hobøl	Linnakoski
<i>O. denti-ciliatum</i>	15748		GQ249331		GQ249321	<i>Betula</i> sp.	Sweden, Filipstad	Roux, Solheim
	19185				GQ249320	<i>Betula</i> sp.	Norway, Tresnes	Kamgan, Solheim
	29493 ^T		FJ804490		FJ804502	<i>S. ratzeburgi</i> ex <i>Betula</i> sp.	Norway, Hobøl	Linnakoski
	29494 ^T		FJ804491		FJ804503	<i>S. ratzeburgi</i> ex <i>Betula</i> sp.	Norway, Hobøl	Linnakoski
	D091318		FJ804492		FJ804504	<i>S. ratzeburgi</i> ex <i>Betula</i> sp.	Norway, Hobøl	Linnakoski
<i>O. borealis</i> sp. nov.	17860		EF408594		GQ249311	<i>T. cordata</i>	Austria	Kirisits
	18893		GQ249325		GQ249312	<i>Betula</i> sp.	Norway, Salangen	Kamgan, Solheim
	18919		EF408592		GQ249313	<i>B. pubescens</i>	Norway, Sørreisa	Kamgan, Solheim
	18941		GQ249326		GQ249314	<i>Betula</i> logs	Norway, Målselv	Kamgan, Solheim
	18953		GQ249327		GQ249315	<i>Betula</i> logs	Norway, Målselv	Kamgan, Solheim
	18956		GQ249328		GQ249316	<i>Betula</i> logs	Norway, Målselv	Kamgan, Solheim
	18966 ^T		EF408593		GQ249317	<i>B. pubescens</i>	Norway, Målselv	Kamgan, Solheim
	19263		GQ249329		GQ249318	<i>Betula</i> logs	Norway, Målselv	Kamgan, Solheim
	19297		GQ249330		GQ249319	<i>Populus</i> sp.	Norway, Sørreisa	Kamgan, Solheim
	19320		EF408591			<i>B. pubescens</i>	Norway, Målselv	Kamgan, Solheim
	23112 ^c		EU443765		EU443778	<i>S. ratzeburgi</i> gallery on <i>Betula</i> sp.	Finland	De Beer
	23113 ^c		EU443765		EU443779	<i>S. ratzeburgi</i> gallery on <i>Betula</i> sp.	Finland	De Beer
						<i>S. ratzeburgi</i> gallery on <i>Betula</i> sp.	Finland	De Beer
						<i>S. ratzeburgi</i> gallery on <i>Betula</i> sp.	Finland	De Beer
						<i>S. ratzeburgi</i> gallery on <i>Betula</i> sp.	Finland	De Beer

^a CMW = Culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.
^b Published sequences of *O. quercus* were assigned to haplotype (HT) groups (numbered alphabetically for ITS and numerically for β -tubulin), each group consisting of identical sequences. Only one sequence per group was deposited in Genbank.
^c Isolates identified as *O. catenatum*-like by Linnakoski *et al.* (2008).
^T Ex-type isolates.

Compilation of sequence data sets

Contigs of both sequenced strands for each isolate were assembled using Sequence Navigator v. 1.01 (ABI PRISM, Perkin Elmer). Additional sequences of related *Ophiostoma* spp., including those from recent studies in Norway and Finland (Linnakoski *et al.*, 2008, 2009), were obtained from GenBank (<http://www.ncbi.nlm.nih.gov>) to fortify the comparisons. Sequences were aligned using the E-INS-i option in the online version of MAFFT 6 (Kato & Toh, 2008). To avoid the inclusion of large numbers of identical sequences in the phylogenetic analyses, ITS and beta-tubulin sequences of *O. quercus* were grouped based on haplotypes using MEGA 4.0.1 (Tamura *et al.*, 2007). ITS haplotype groups were labeled A-H and the beta-tubulin haplotype groups 1-16. From each haplotype group, only one isolate was included in the analyses.

Phylogenetic analyses

Phylogenetic analyses of the ITS and beta-tubulin data sets were performed independently of each other, because reference sequences were not available for the same sets of isolates. For each data set, maximum parsimony (MP), Bayesian analyses (MB), and maximum likelihood (ML) analyses were done.

MP analyses were performed in similar manner for both data sets in PAUP 4.0b10 (Swofford, 1998). Only parsimony informative characters (196 for ITS and 122 for beta-tubulin) were included, using the following settings: 100 random sequence addition replicates, tree bisection-recognition (TBR) branch swapping, and 'multrees' option in effect. Confidence levels of the MP phylogenies were estimated with the bootstrap method (1000 replications).

Bayesian analyses based on Markov chain Monte Carlo (MCMC) were performed with MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001). Ten million generations were run in four chains with sampling every 100 generations, which yielded 100 000 trees. Appropriate substitution models were determined using the Akaike Information Criterion (AIC) in MrModeltest 2.2 (<http://www.abc.se/~nylander/>). The model applied to the ITS region was GTR+I+G and for the beta-tubulin GTR+G was used. Burn-in values were determined using Tracer 1.4 (<http://beast.bio.ed.ac.uk/Tracer>). For ITS the first 500 trees were discarded as burn-in and for beta-tubulin the first 1000 trees.

ML analyses were conducted online using PhyML 3.0 (Guindon & Gascuel, 2003). The AIC was used in Modeltest 3.7 (Posada & Crandall, 1998) to select appropriate substitution models for the two data sets. For ITS, the selected model was GTR+I+G (proportion of invariable sites = 0.3779; gamma shape parameter = 0.6576) and for beta-tubulin it was GTR+G (gamma shape parameter = 0.2000).

RESULTS

Collection of isolates

In total, 230 cultures were obtained from bark and cambial samples collected from more than 200 logs, stumps and stems of broad-leaved tree species from five different native tree genera in Norway and Sweden. Most collections

were obtained from *Betula* spp. (121) and *Quercus* spp. (80), while ophio-stomatoid fungi was also obtained from *Populus tremula*, *Sorbus aucuparia* and *Salix* spp.

Morphology of cultures

Most isolates obtained produced only *Pesotum* anamorphs, while only a few produced both ascomata and synnemata in culture. Based on the cultural variation, a total of 73 isolates including representative strains from different tree genera, different geographical locations sampled and different morpho-groups were selected for DNA sequencing (Table 2) to obtain an indication of the species diversity within the samples collected in Norway and Sweden.

DNA sequencing and phylogenetic analyses

All isolates selected for DNA sequencing produced fragments of approximately 650 bp using the primers ITS1 and ITS4, while the beta-tubulin fragments were about 500 bp in size. Alignment of sequences for isolates from Norway (Table 1) with related sequences from GenBank resulted in a total of 683 characters including gaps, with 403 constant characters, 84 parsimony-uninformative characters and 196 parsimony informative characters for the ITS data set. For the beta-tubulin data set 343 characters including gaps, with 204 constant characters, 17 parsimony-uninformative characters and 122 parsimony informative characters were obtained. MP analyses resulted in 64 trees of 475 steps for ITS, and 1512 trees of 446 steps for beta-tubulin data sets. For the respective data sets the consistency indexes (CI) were 0.747 and 0.608, while the retention indexes (RI) values were 0.864 and 0.901. For each data set a 50% majority rule tree obtained from Bayesian analyses is presented (Figs 1 and 2). Bayesian posterior probabilities $\geq 90\%$, and MP and ML bootstrap values $\geq 75\%$, are indicated at the relevant nodes.

Table 2. Isolates of *Ophiostoma* identified as part of the present study from broad-leaved hosts in different parts of Norway and Sweden (underlined) during surveys in 2004 and 2005.

Host Tree	<i>O. quercus</i>	<i>O. borealis</i>	<i>O. denticiatum</i>
<i>Betula</i>	^a <u>15608</u> , 15528, 18878, 18883, 18939, 19265, 19266, 19267, 19270, 19272, 19276, 19277, 19285, 19287, 19296, 19314, 19317, 22055, 22066, 22070, 22072	18893, 18919, 18941, 18953, 18956, 18966, 19263, 19320	<u>15748</u> , 19185
<i>Populus</i>	15592, 18917, 19175, 19178, 19182, 19183, 19184, 19192, 19193, 19254, 19258	19297	
<i>Quercus</i>	15704, 15746, 15750, 19187, 19198, 19201, 19212, 19214, 19222, 19223, 19240, 19241, 19242, 19243, 19244, 19247, 19248, 19250, 19251, 19252, 19253, 19255, 19256, 19257, 19261		
<i>Salix</i>	19260, 19264		
<i>Sorbus</i>	15544, 15560, 15566		

^aAll numbers represent CMW numbers if cultures in the Culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

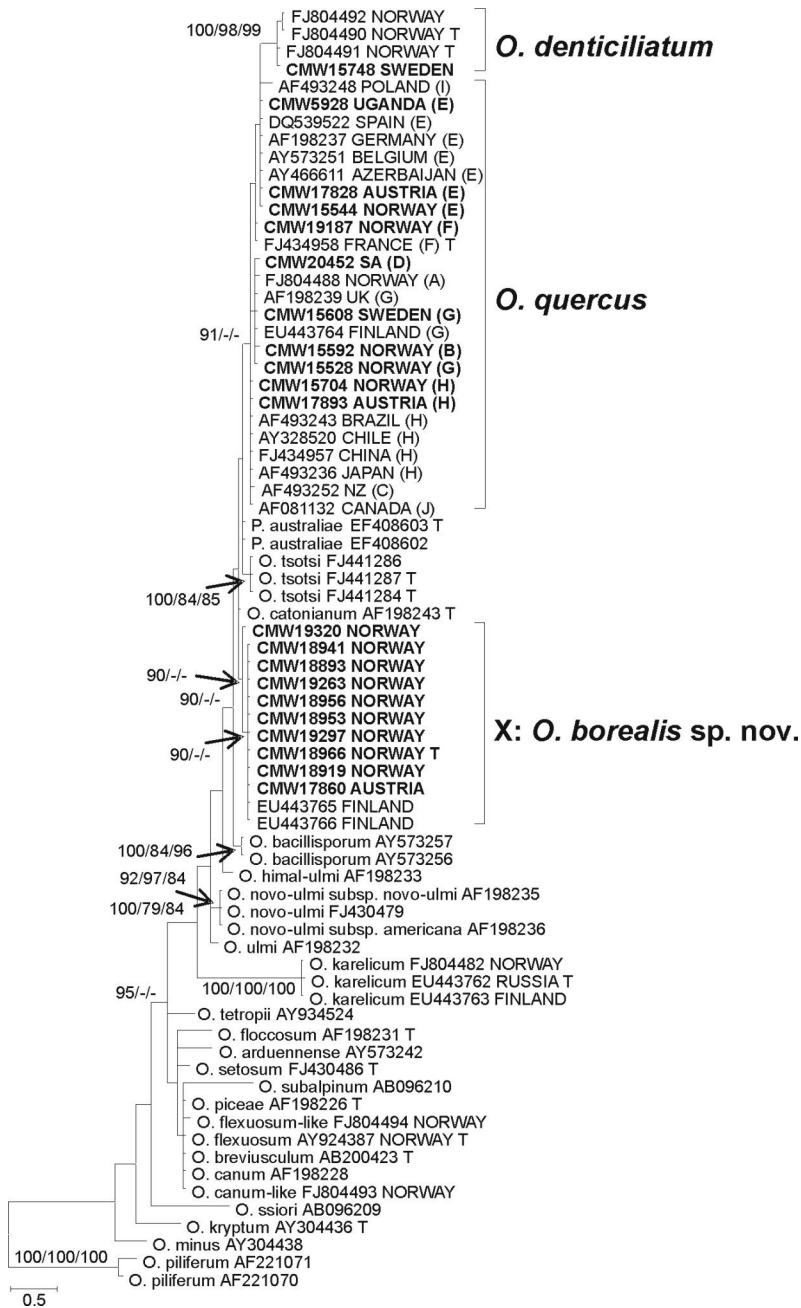


Fig. 1. Phylogenetic tree produced by a Bayesian analysis of the ITS sequence data. *Ophiostoma piliferum* was used as outgroup taxon. Posterior probabilities above 90% are presented at nodes, followed by bootstrap values above 75% for respectively MP and ML analyses. Isolates sequenced in this study are printed in bold type. Sequences of *O. quercus* isolates were assigned to haplotype groups, indicated as (A-J). T = ex-type isolates.

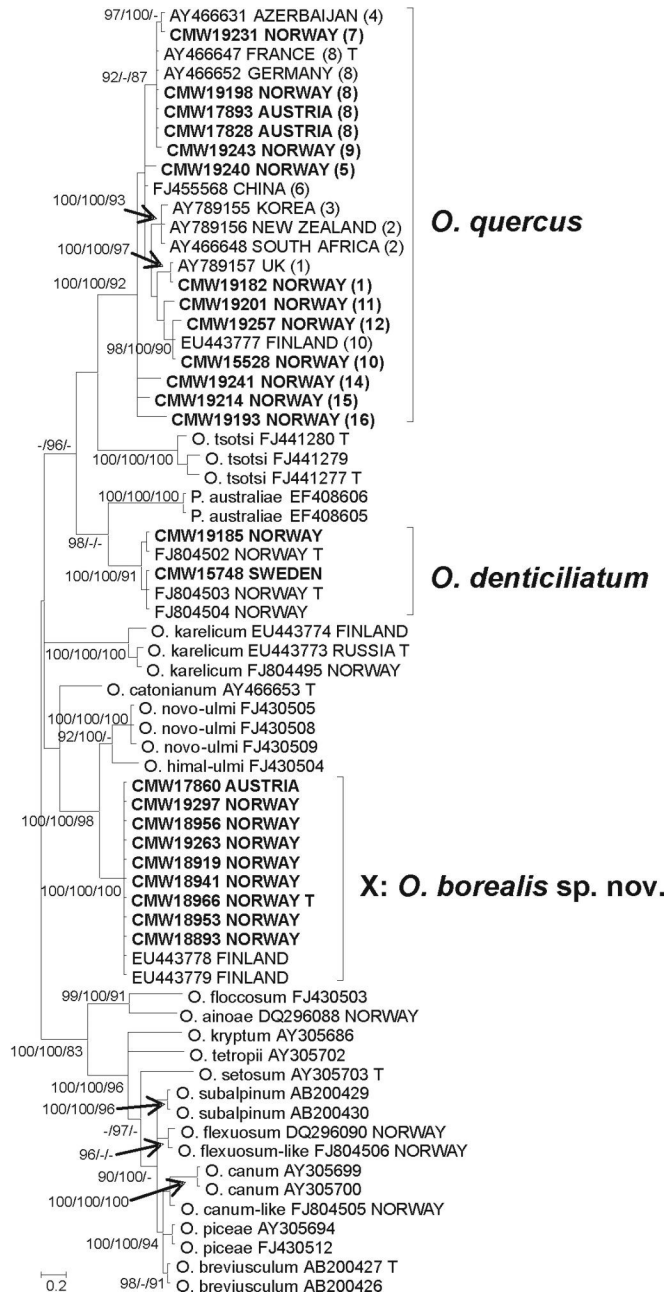


Fig. 2. A midpoint-rooted phylogenetic tree produced by a Bayesian analysis of the beta-tubulin sequence data. Posterior probabilities above 90% are presented at nodes, followed by bootstrap values above 75% for respectively MP and ML analyses. Isolates sequenced in this study are printed in bold type. Sequences of *O. quercus* isolates were assigned to haplotype groups, indicated as (1-16). T = ex-type isolates.

Based on ITS analyses (Fig. 1), most of the Norwegian isolates selected for sequencing grouped with *O. quercus* isolates from previous studies. The Norwegian *O. quercus* isolates belonged to haplotypes A, B, E, F, G, H and the Swedish isolate to haplotype G (Table 1). A single isolate from Sweden (CMW 15748) grouped with *O. denticiliatum* isolates from *Scolytus ratzeburgii* on birch in Norway (Linnakoski *et al.* 2009). Nine Norwegian and one of the reference isolates from Austria, that were considered to be *O. quercus*, formed a lineage (X in Fig. 1) distinct from *O. quercus*. Lineage X also included two isolates from *S. ratzeburgii* galleries on birch in Finland, referred to as *O. catonianum*-like by Linnakoski *et al.* (2008).

Analyses of the beta-tubulin data (Fig. 2) gave results similar to those obtained with ITS. The Norwegian *O. quercus* isolates belonged to haplotypes 1, 5, 7, 8, 9, 10, 11, 12, 14, 15 and 16, and the Swedish isolate to haplotype 10 (Tab. 1). The *O. quercus* lineage showed substantial variability within the group, but with very high statistical support delineating the species clearly from its closest relative, *O. tsotsi* Grobbelaar, Z.W. de Beer & M.J. Wingf. (Grobbelaar *et al.* 2010). Beta-tubulin sequence data also showed that in addition to the Swedish isolate (CMW 15748) grouping with *O. denticiliatum* in the ITS analysis, a Norwegian isolate from birch (CMW 19185) also belonged to this species. The same group of isolates constituting lineage X in the ITS analysis, formed a strongly supported group closest to, but clearly distinct from the Dutch elm disease fungi and *O. catonianum*.

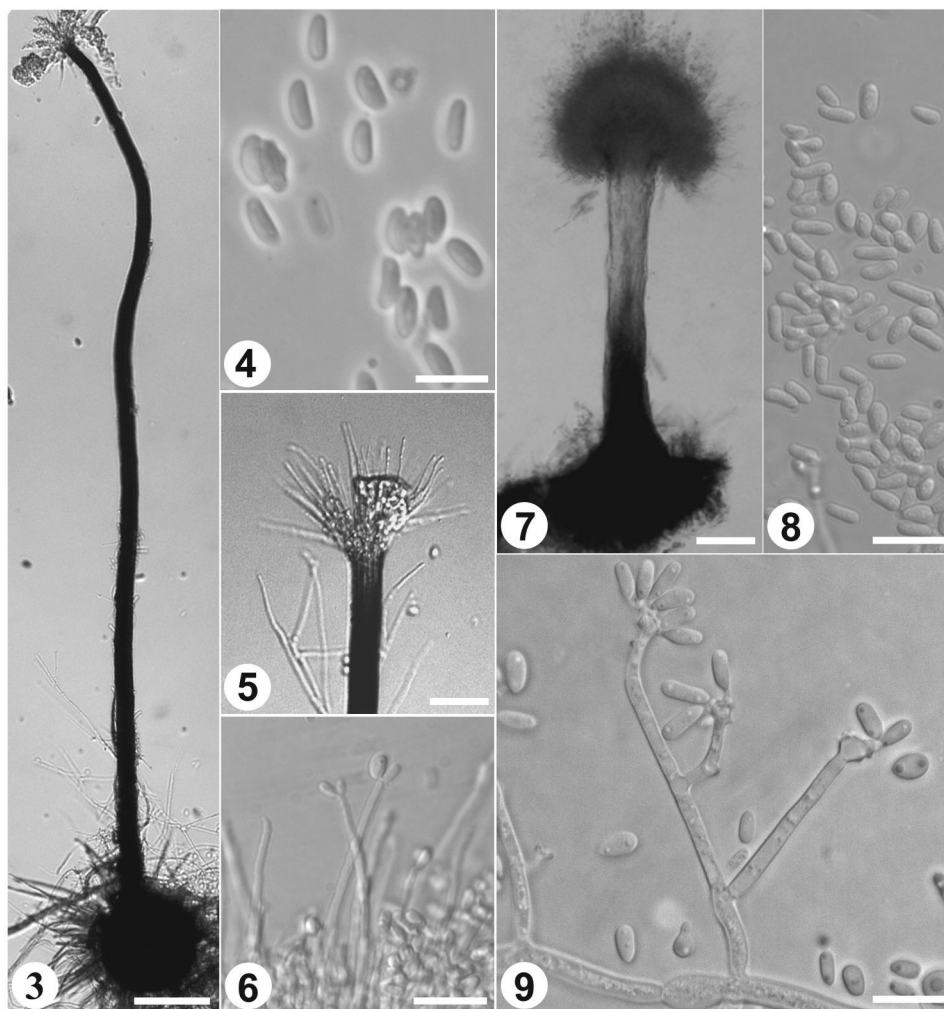
TAXONOMY

Phylogenetic analyses of the ITS (Fig. 1) and partial beta tubulin (Fig. 2) gene regions, revealed that several isolates from Norway and Austria represented an undescribed taxon (lineage X). This was further supported by morphological comparisons and a new species is, therefore, described as follows:

***Ophiostoma borealis* sp. nov.** G. Kamgan Nkuekam, H. Solheim & Z.W. de Beer-
MB 518853 Figs 3-9

Etymology: The species name refers to the boreal ecosystem where the fungus was collected.

Coloniae in OMA olivaceo-griseae vel fumeae, in MEA bubalinae vel melleae, in MEA ad 25°C ad 41 mm in 10 diebus crescentes. Optime crescunt ad 20°C. *Bases* ascomatae nigrae globosae (74-) 103-175 (-274) µm longae, (72-) 103-170 (-233) µm latae, cum trichomatibus hypharum; *colla* nigra (295-) 341-1041 (-1828) µm longa. *Hyphae* ostiolorum divergentes (17-) 26-48 (-58) µm longae. *Ascosporae* reniformes, non septatae (3.4-) 3.8-4.3 (-4.6) µm longae, (1.4-) 1.5-1.9 (-2.1) µm latae. Anamorphae biformes. Anamorpha *Pesotum* conidiophoris erectis atrobrunneis apicem versus pallescentibus (82-) 129-212 (-246) µm longis. *Capitula conidiogena* maxime (33-) 48-77 (-100) µm lata, laete brunnea, apicem versus hyalinescentia. *Cellulae conidiogenae* hyalinae acerosae (5-) 14-30 (-44) µm longae, (0.5-) 1-1.7 (-2.2) µm latae. *Conidia* non septata, hyalina, oblonga vel obovoidea (3.8-) 4-5.7 (-6.9) µm × (1-) 1.8-2 (-2.8) µm. Conidiophorae *Sporothrix* hyalinae (15-) 19-45 (-77) × (1-) 1.8-2.4 (-2.7) µm cum denticulis prominentis.



Figs 3-9. Morphological characteristics of *Ophiostoma borealis* sp. nov. (CMW 18966). Teleomorph: **3**, globose ascomatal base (scale bar = 50 µm), **4**, reniform ascospores (scale bar = 10 µm), **5**, divergent ostiolar hyphae (scale bar = 20 µm). *Pesotum* anamorph: **6**, conidiogenous cells with conidia at the tips of sympodially proliferating conidiogenous cells (scale bar = 10 µm), **7**, synnemata (scale bar = 20 µm) showing hyphal hairs at base, **8**, oblong to cylindrical conidia (scale bar = 10 µm). *Sporothrix* anamorph: **9**, conidiogenous cell with emerging conidia (scale bar = 10 µm).

Conidia non septata, hyalina, obovoidea, interdum manifeste acerosa (7.6-) 8.9-11.3 (-13.8) × (2.9-) 3.2-4 (-4.8) µm.

Teleomorph state produced on *Betula pubescens* chips after random crossing of strains after approximately two months. *Ascomatal* bases black, globose (74-) 103-175 (-274) µm long and (72-) 103-170 (-233) µm wide with hyphal hairs. *Ascomatal* necks black (295-) 341-1041 (-1828) µm long, base of necks smooth (22-) 32-53 (-69) µm wide, middle of necks (15-) 18-26 (-35) µm

wide, tips of necks (9-) 13-18 (-25) μm wide. *Ostiolar hyphae* present, divergent (17-) 26-48 (-58) μm long. *Asci* evanescent. *Ascospores* reniform in side view, aseptate (3.4-) 3.8-4.3 (-4.6) μm long and (1.4-) 1.5-1.9 (-2.1) μm wide, accumulating in round, creamy spore drops.

Colonies Olivaceous grey (21''''i) or smoke grey (21''''d) on OMA with conidiophores forming light-colored slimy heads, becoming creamy toward the centers and arranged in mostly circular rings, reverse mouse grey (15''''d) to almost black. On MEA colonies buff (19''d) with conidiophores forming cream-colored slimy heads scattered over the colony mostly surrounded by mat of mycelia, reverse colonies buff (19''d) becoming honey (19''b) toward the middle of the plate. Colony diameter reaching 41 mm in 10 days on MEA at 25 °C. Optimal growth temperature 20 °C, no growth at 5 °C or at 35 °C.

Pesotum anamorph: *Conidiophores* synnematal, erect, dark brown at the bases, becoming lighter towards the apex, (82-) 129-212 (-246) μm long, (9-) 13-21 (-30) μm wide in the middle, (16-) 20-31 (-38) μm wide at the base. *Conidiogenous heads* (33-) 48-77 (-100) μm across the widest part, light brown becoming hyaline towards the apex. *Conidiogenous cells*, hyaline, acerose (5-) 14-30 (-44) μm long, (0.5-) 1-1.7 (-2.2) μm wide, tapering towards the apex. *Conidia* produced through holoblastic, sympodial development. *Conidia* aseptate, hyaline, oblong to obovoid, accumulating in slimy heads on the apices of the synnemata, (3.8-) 4-5.7 (-6.9) $\mu\text{m} \times$ (1-) 1.8-2 (-2.8) μm .

Sporothrix anamorph: conidiophores hyaline, cylindrical and branched, tapering towards the apex, (15-) 19-45 (-77) \times (1-) 1.8-2.4 (-2.7) μm , prominent denticles present. *Conidia*, aseptate, hyaline, obovoid, distinctly acerose in some cases (7.6-) 9-11.3 (-13.8) \times (3-) 3.2-4 (-4.8) μm .

Specimens examined: Norway, Troms, Målselv, Rostadalen, Dalhaug, isolated from wounds on logs of newly felled *Betula pubescens*. June 2005, G. Kamgan Nkuekam & H. Solheim, holotype PREM 59745, living culture CMW 18966, CBS 123222, NFLI 05-703/2.

Additional specimens: Norway, Troms, Målselv, but from three different sites (or localities); Dalhaug in Rostadalen, Moeng near Øverbygd and Sandbakken near Olsborg, isolated from wounds on logs of newly felled *B. pubescens*. June 2005, G. Kamgan Nkuekam & H. Solheim, paratypes, PREM 59742 (Living cultures CMW 18953, CBS 123220, NFLI 05-677/2); PREM 59747 (Living cultures CMW 18919, CBS 123221, NFLI 05-605/2); PREM 59744 (Living cultures CMW 18956, NFLI 05-689); PREM 59746 (Living cultures CMW 18941, NFLI 05-659/2).

DISCUSSION

Results of this study have shown that there is a relatively low diversity of *Ophiostoma* spp. on hardwoods in Norway. More than 200 isolates were obtained in a fairly limited study period, showing for the first time that *Ophiostoma* spp. are common on native hardwood trees in Norway. Because of the large number of isolates collected, and because it is notoriously difficult to identify *Ophiostoma* spp., such as those in the *O. piceae* complex (Harrington *et al.*, 2001), isolates were subjected to a preliminary screening based primarily on culture morphology. Representative isolates were selected for each morphological group and the

resulting 73 isolates were further characterized based on DNA sequence comparisons. This resulted in the identification of three distinct *Ophiostoma* spp.: *O. borealis* sp. nov., *O. denticiliatum* and *O. quercus*.

This study resulted in the discovery of a new *Ophiostoma* sp., *O. borealis* based on sequence analyses. The fungus groups in the hardwood inhabiting clade of the *O. piceae* complex and is closely related to *O. catonianum* and *O. novo-ulmi*. The fact that *O. borealis* was collected from Northern Norway and also identified from cultures from Austria and sequences from Finland (Linnakoski *et al.*, 2008), suggests that this species has a wide distribution in Europe. Although the majority of isolates originated from *Betula*, its presence on *Populus* in Norway and *Tilia cordata* Mill. in Austria suggests that it has a broad host range. Given the difficulty in distinguishing between the synnematal anamorphs of *O. borealis* and other *Ophiostoma* spp., it is possible that *O. borealis* has been recorded in Europe before, but under the names *O. piceae* or *O. quercus*.

Morphological differences between *O. borealis* and its closest relatives, *O. quercus*, *O. novo-ulmi* and *O. catonianum*, are difficult to define due to variation in the dimensions of structures often present within a single culture. However, when ranges of dimensions are compared (Table 3), some differences between the species are evident. The most notable of these is that *O. borealis* produces substantially shorter synnemata than the other species, although length ranges overlap with *O. catonianum*. *Ophiostoma quercus* can be distinguished from *O. borealis* and the other two species by the presence of very long conidia produced by its *Sporothrix* anamorph. Where *O. borealis* and *O. quercus* occur on a variety of host tree species, *O. novo-ulmi* and *O. catonianum* have been recorded only from *Ulmus* and *Pyrus* spp, respectively. *Ophiostoma borealis*, *O. quercus* and *O. novo-ulmi* are heterothallic, while *O. catonianum* is homothallic. Furthermore, in the illustrations of Goidánich (1935), *O. catonianum* produces allantoid ascospores similar to the other species, but with ends slightly more pointed.

Table 3. Comparison of the most relevant morphological features distinguishing *O. borealis* sp. nov. from *O. quercus*, *O. novo-ulmi* and *O. catonianum*. All measurements in µm.

	<i>O. borealis</i> sp. nov. (present study)	<i>O. quercus</i> (Grobbelaar <i>et al.</i> 2009; Halmschlager <i>et al.</i> 1994)	<i>O. novo-ulmi</i> (Brasier 1991)	<i>O. catonianum</i> (Goidánich 1935)
Perithecia:				
neck length	295-1827	306-2036	230-1070	475-630
base width	72-232	110-217	75-140	110-165
Ascospore: length				
width	3.4-4.6	2.6-4.8	4.5-6	3-5
Pesotum stage:				
synnemata	1.4-2.1	0.9-2.4	1-1.5	1.5-1.8
conidia	82-260	227-1097	< 2000	26-550
	3.8-6.9 × 1-2.8	2.5-4.8 × 1-2.4	2-6 × 1-3	3-5 × 1.6-2
Sporothrix stage:				
conidia	7.6-13.8	14-233	4.5-14	10-16
Thallism	heterothallic	heterothallic	heterothallic	homothallic
Host range	hardwoods	hardwoods	restricted to <i>Ulmus</i> spp.	only known from <i>Pyrus</i> sp.

Of the *Ophiostoma* spp. identified, *O. quercus* was by far the most common. This fungus has previously been reported only from the birch bark beetle at a single site in Norway. However, the report of *O. piceae* as common in decayed *Betula* logs in Norway (Venn, 1972) probably more appropriately reflects *O. quercus*. Results of this study considerably extend the host range and distribution of *O. quercus* to five genera of hardwood hosts spread over all the major vegetation zones in Norway.

It was not surprising to find *O. quercus* on hardwood trees in this study. The fungus is well-known to have a wide distribution in the northern hemisphere on deciduous timber, although many reports have been obscured by taxonomic confusion. Many early reports of the fungus on hardwoods were of *O. piceae* and it is only relatively recently that *O. piceae* isolates from hardwoods have been recognized as most probably representing the distinct fungus, *O. quercus* (Morelet, 1992; Brasier & Kirk, 1993; Halmschlager *et al.*, 1994; Kim *et al.*, 1999). Some authors have also referred to the fungus as *O. querci* which might have confused the literature pertaining to it even further (De Beer *et al.*, 2003a). Interestingly, the fungus has a cosmopolitan distribution on hardwoods and is less commonly found on conifers. It has been reported from native and non-native trees in North America (Kim *et al.*, 1999; Juzwik *et al.*, 1998), South America (Harrington *et al.*, 2001; De Beer *et al.*, 2003b; Geldenhuis *et al.*, 2004; Zhou *et al.*, 2004), Europe (Morelet, 1992; Brasier & Kirk, 1993; Halmschlager *et al.*, 1994; Carlier *et al.*, 2006; Romon *et al.*, 2007; Grobbelaar *et al.*, 2009), Africa (De Beer *et al.*, 2003b; Zhou *et al.*, 2006; Kamgan Nkuekam *et al.*, 2008a, b), East Asia (Kim *et al.*, 2005; Masuya *et al.*, 2009; Paciura *et al.*, 2010) and Australasia (Harrington *et al.*, 2001; Thwaites *et al.*, 2005). It is thus not unusual to find the fungus commonly associated with wounds on broad-leaved trees in Norway, and also in Austria and Sweden.

Ophiostoma denticiliatum was isolated only twice in this study, on both occasions from birch. The species was first isolated and described from the birch bark beetle, *Scolytus ratzeburgi*, in Norway (Linnakoski *et al.*, 2009). Interestingly, it was not isolated from the galleries of the beetle in that study, and neither was it found in a previous study of the beetle and its associated fungi in Finland and Russia (Linnakoski *et al.*, 2008). It is thus unlikely to be an obligate associate of the beetle, but might be associated with hyperphoretic mites as has been found for many other *Ophiostoma* spp. (Bridges & Moser, 1983; Moser *et al.*, 1989). Linnakoski *et al.* (2009), reported that *O. denticiliatum* cultures reach only 35 mm in 10 days and this is substantially slower than *O. quercus* cultures that reach 41–57 mm in the same period (Grobbelaar *et al.*, 2009). This is in contrast to *O. borealis* cultures that reach 41 mm in ten days and thus only the lower end of the growth range for *O. quercus*. Because of its aggressive growth, *O. quercus* might easily out-compete *O. borealis* and more so, *O. denticiliatum*, on exposed sapwood surfaces. This could explain why fewer isolates of the latter species were collected in this study.

Linnakoski *et al.* (2008, 2009), recorded seven *Ophiostoma* spp. from the birch bark beetle and its galleries and these resulted from relatively limited collections. In contrast, the present study included relatively extensive sampling but resulted in only three *Ophiostoma* spp. being found. This, surprisingly limited diversity might reflect the sampling strategy that would have favoured the rapidly growing and apparently aggressive *O. quercus*. In contrast, if isolations from wound-visiting arthropods had been included, a greater diversity of these fungi might have emerged.

This study has improved our knowledge about the occurrence, host range and geographic distribution of *Ophiostoma* spp. in Norway. *Ophiostoma quercus* is without doubt a widespread species occurring on a wide range of substrates as confirmed again in this study. *Ophiostoma borealis*, although only representing nine of the sequenced isolates, could be more widespread than indicated, due to the rough screening used to select isolates for DNA sequence comparisons. However, this study has clearly shown that there is much to be learned regarding the occurrence of *Ophiostoma* spp. in Norway, and this is despite the fact that this is one of the best studied fungal genera in the world.

Acknowledgements. We thank the Department of Science and Technology (DST), Center of Excellence in Tree Health Biotechnology (CTHB), National Research Foundation of South Africa (NRF), the THRIP Initiative of the Department of Trade and Industry (THRIP/DST), Norwegian Research Council (NFR), Norwegian Forest and Landscape Institute (NFLI), members of the Tree Protection Co-operative Programme (TPCP) and the University of Pretoria for funding and the facilities to undertake this study. We also thank Prof. Paal Krokene for hosting the first, fourth and last author during research visits in Norway, the forest officers Brynjar Jørgensen, Trude Hagen Hansen, Terje Dahl and Geir Kvammen for all help during the stay in Troms, and likewise Prof. Thomas Kirisits for providing Austrian isolates. Dr. Hugh Glen is acknowledged for providing the Latin translations.

REFERENCES

- BRASIER C.M., 1991 — *Ophiostoma novo-ulmi* sp. nov., causative agent of current Dutch elm disease pandemics. *Mycopathologia* 115: 151-161.
- BRASIER C.M., 2000 — Intercontinental spread and continuing evolution of the Dutch elm disease pathogens. In: Dunn CP (eds), *The elms: breeding, conservation and disease management*. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 61-72.
- BRASIER C.M. & KIRK S.A., 1993 — Sibling species within *Ophiostoma piceae*. *Mycological Research* 97: 811-816.
- BRIDGES R.J. & MOSER J.C., 1983 — Role of two phoretic mites in transmission of bluestain fungus, *Ceratocystis minor*. *Ecological Entomology* 8: 9-12.
- CARLIER F.X., DECOCK C., JACOBS K. & MARAITE H., 2006 — *Ophiostoma arduennense* sp. nov. (Ophiostomatales, Ascomycota) from *Fagus sylvatica* in southern Belgium. *Mycological Research* 110: 801-810.
- DE BEER Z.W., GLEN H.F., WINGFIELD B.D. & WINGFIELD M.J., 2003a — *Ophiostoma quercus* or *Ophiostoma querci*? *Mycotaxon* 136: 211-214.
- DE BEER Z.W., WINGFIELD B.D. & WINGFIELD M.J., 2003b — The *Ophiostoma piceae* complex in the southern hemisphere: a phylogenetic study. *Mycological Research* 107: 469-476.
- FRIES E., 1823 — Ordines, genera et species, huc usque cognitae. *Systema Mycologicum* 2: 472-473.
- GELDENHUIS M.M., ROUX J., MONTENEGRO F., DE BEER Z.W., WINGFIELD M.J. & WINGFIELD B.D., 2004 — Identification and pathogenicity of *Graphium* and *Pesotum* species from machete wounds on *Schizolobium parahybum* in Ecuador. *Fungal Diversity* 15: 135-149.
- GIBBS J.N., 1980 — Role of *Ceratocystis piceae* in preventing infection by *Ceratocystis fagacearum* in Minnesota. *Transactions of the British Mycological Society* 74: 171-174.
- GLASS N.L. & DONALDSON G.C., 1995 — Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous Ascomycetes. *Applied and Environmental Microbiology* 61: 1323-1330.
- GOIDÁNICH G., 1935 — Una nuova specie di *Ophiostoma* vivente sul pero ed alcune osservazioni sull'estta posizione sistematica della forma ascofora e delle forme metagenetiche del genere. *Bollettino della Stazione di Patologia Vegetale di Roma* 15: 122-168.

- GROBBELAAR J., AGHAYEVA D., DE BEER Z.W., BLOOMER P., WINGFIELD M.J. & WINGFIELD B.D., 2009 — Delimitation of *Ophiostoma quercus* and its synonyms using multiple gene phylogenies. *Mycological Progress* 8: 221-236.
- GROBBELAAR J., DE BEER Z.W., BLOOMER P., WINGFIELD M.J. & WINGFIELD B.D., 2010 — *Ophiostoma tsotsi* sp. nov., a wound-infesting fungus of hardwood trees in Africa. *Mycopathologia* 169: 413-423.
- GUINDON S. & GASCUEL O., 2003 — A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology* 52: 696-704.
- HALMSCHLAGER E., MESSNER R., KOWALSKI T. & PRILLINGER H., 1994 — Differentiation of *Ophiostoma piceae* and *Ophiostoma quercus* by morphology and RADP analysis. *Systematic and Applied Microbiology* 17: 554-562.
- HARRINGTON T.C., 2005 — Ecology and evolution of mycophagous bark beetles and their fungal partners. In: Vega FE, Blackwell M (eds.), *Insect-fungal associations: Ecology and Evolution*. Oxford University Press, New York, pp. 1-22.
- HARRINGTON T.C., MCNEW D., STEIMEL J., HOFSTRA D. & FARRELL R., 2001 — Phylogeny and taxonomy of the *Ophiostoma piceae* complex and the Dutch elm disease fungi. *Mycologia* 93: 111-136.
- HUELSENBECK J.P. & RONQUIST F., 2001 — MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* 17: 754.
- JUZWIK J., CEASE K.R. & MEYER J.M., 1998 — Acquisition of *Ophiostoma quercus* and *Ceratocystis fagacearum* by nitidulids from *O. quercus*-colonized oak wilt mats. *Plant Disease* 82: 239-243.
- JUZWIK J. & FRENCH D.W., 1983 — *Ceratocystis fagacearum* and *C. piceae* on the surfaces of free-flying and fungus-mat-inhabiting nitidulids. *Phytopathology* 73: 1164-1168.
- KAMGAN NKUEKAM G., JACOBS K., DE BEER Z.W., WINGFIELD M.J. & ROUX J., 2008a — *Ceratocystis* and *Ophiostoma* species including three new taxa, associated with wounds on native South African trees. *Fungal Diversity* 29: 37-59.
- KAMGAN NKUEKAM G., JACOBS K., DE BEER Z.W., WINGFIELD M.J. & ROUX J., 2008b — *Pesotum australi* sp. nov. and *Ophiostoma quercus* associated with *Acacia mearnsii* trees in Australia and Uganda, respectively. *Australasian Plant Pathology* 37: 406-416.
- KATOH K. & TOH H., 2008 — Recent developments in the MAFFT multiple sequence alignment program. *Briefings in Bioinformatics* 9: 286-298.
- KIM G-H., KIM J-J., LIM Y.W. & BREUIL C., 2005 — Ophiostomatoid fungi isolated from *Pinus radiata* logs imported from New Zealand to Korea. *Canadian Journal of Botany* 83: 272-278.
- KIM S.H., UZUNOVIC A. & BREUIL C., 1999 — Rapid detection of *Ophiostoma piceae* and *O. quercus* in stained wood by PCR. *Applied Environmental Microbiology* 65: 287-290.
- LAGERBERG T., LUNDBERG G. & MELIN E., 1927 — Biological and practical researches into blueing in pine and spruce. *Svenska Skogsvårdsföreningens Tidskrift* 25: 145-272.
- LINNAKOSKI R., DE BEER Z.W., ROUSI M., NIEMELÄ P., PAPPINEN A. & WINGFIELD M.J., 2008 — Fungi including *Ophiostoma karelicum* sp. nov., associated with *Scolytus ratzeburgi* infesting birch in Finland and Russia. *Mycological Research* 112: 1475-1488.
- LINNAKOSKI R., DE BEER Z.W., ROUSI M., SOLHEIM H. & WINGFIELD M.J., 2009 — *Ophiostoma denticiliatum* sp. nov. and other *Ophiostoma* species associated with the birch bark beetle in southern Norway. *Persoonia* 23: 9-15.
- LOGAN J.A., RÉGNIÈRE J. & POWELL J.A., 2003 — Assessing the impacts of global warming on forest pest dynamics. *Frontiers in Ecology and the Environment* 1: 130-137.
- MASUYA H., YAMAOKA Y., KANEKO S. & YAMAURA Y., 2009 — Ophiostomatoid fungi isolated from Japanese red pine and their relationships with bark beetles. *Mycoscience* 50: 212-223.
- MATHIESEN A., 1950 — Über einige mit Borkenkäfern assoziierte Bläuepilze in Schweden. *Oikos* 2: 275-308.
- MATHIESEN A., 1951 — Einige neue *Ophiostoma*-Arten in Schweden. *Svensk Botanisk Tidskrift* 45: 203-232.
- MATHIESEN-KÄÄRIK A., 1953 — Eine Übersicht über die gewöhnlichsten mit Borkenkäfern assoziierten Bläuepilze in Schweden und einige für Schweden neue Bläuepilze. *Meddelanden från Statens Skogsforskningsinstitut* 43: 1-74.
- MATHIESEN-KÄÄRIK A., 1960 — Studies on the ecology, taxonomy and physiology of Swedish insect-associated blue stain fungi, especially the genus *Ceratocystis*. *Oikos* 11: 1-25.
- MELIN E. & NANNFELDT J.A., 1934 — Researches into the blueing of ground wood-pulp. *Svenska Skogsvårdsföreningens Tidskrift* 32: 397-616.

- MÖLLER E.M., BAHNWEG G., SANDERMANN H. & GEIGER H.H., 1992 — A simple and efficient protocol for isolation of high molecular weight DNA from filamentous fungi, fruit bodies, and infected plant tissues. *Nucleic Acids Research* 20: 6115-6116.
- MORELET M., 1992 — *Ophiostoma querci* sur chêne en France. *Annales de la Société des Sciences Naturelles et d'Archéologie de Toulon et du Var* 44: 106-112.
- MOSER J.C., PERRY T.J. & SOLHEIM H., 1989 — Ascospores hyperphoretic on mites associated with *Ips typographus*. *Mycological Research* 93: 513-517.
- O'DONNELL K. & CIGELNIK E., 1997 — Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. *Molecular Phylogenetics and Evolution* 7: 103-116.
- PACIURA D., ZHOU X., DE BEER Z.W., JACOBS K., YE H. & WINGFIELD M.J., 2010 — Characterisation of synnematus bark beetle-associated fungi from China, including *Graphium carbonarium* sp. nov. *Fungal Diversity* 40: 75-88.
- POSADA D. & CRANDALL K.A., 1998 — MODELTEST: testing the model of DNA substitution. *Bioinformatics* 14: 817-818.
- RAFFA K.F., AUKEMA B.H., BENTZ B.J., CARROLL A.L., HICKE J.A., TURNER M.G. & ROMME W.H., 2008 — Cross-scale drivers of natural disturbances prone to anthropogenic amplification: the dynamics of bark beetle eruptions. *BioScience* 58: 501-517.
- RAYNER R.W., 1970 — *A mycological colour chart*. Kew, Surrey, Commonwealth Mycological Institute and British Mycological Society.
- ROBAK H., 1932 — Investigations regarding fungi on Norwegian ground wood pulp and fungal infection at wood pulp mills. *Nyt Magazin for Naturvidenskaberne* 71: 185-330.
- ROLL-HANSEN F., 1985 — Outbreaks and new records. Norway. *Ceratocystis ulmi* on *Ulmus glabra*. *FAO. Plant Protection Bulletin* 33: 75.
- ROLL-HANSEN F. & ROLL-HANSEN H., 1980a — Microorganisms which invade *Picea abies* in seasonal stem wounds. I. General aspects. Hymenomycetes. *European Journal of Forest Pathology* 10: 321-339.
- ROLL-HANSEN F. & ROLL-HANSEN H., 1980b — Microorganisms which invade *Picea abies* in seasonal stem wounds. II. Ascomycetes, Fungi imperfecti, and Bacteria. General discussion, Hymenomycetes included. *European Journal of Forest Pathology* 10: 396-410.
- ROMÓN P., ZHOU X., ITURRONDORBEITIA J.C., WINGFIELD M.J. & GOLDARAZENA A., 2007 — *Ophiostoma* species (Ascomycetes: Ophiostomatales) associated with bark beetles (Coleoptera: Scolytinae) colonizing *Pinus radiata* in northern Spain. *Canadian Journal of Microbiology* 53: 756-767.
- SIX D.L., 2003 — Bark beetle fungus symbiosis. In: Bourtzis K, Miller TA (eds), *Insect symbiosis*. CRC Press, New York, pp. 97-114.
- SKARPAAS O. & ØKLAND B., 2009 — Timber import and the risk of forest pest introductions. *Journal of Applied Ecology* 46: 55-63.
- SOLHEIM H., 1986 — Species of Ophiostomataceae isolated from *Picea abies* infested by the bark beetle *Ips typographus*. *Nordic Journal of Botany* 6: 199-207.
- SOLHEIM H., 1992a — Fungal succession in sapwood of Norway spruce infested by the bark beetle *Ips typographus*. *European Journal of Forest Pathology* 22: 136-148.
- SOLHEIM H., 1992b — The early stages of fungal invasion in Norway spruce infested by the bark beetle *Ips typographus*. *Canadian Journal of Botany* 70: 1-5.
- SOLHEIM H., 1993 — Fungi associated with the spruce bark beetle *Ips typographus* in an endemic area in Norway. *Scandinavian Journal of Forest Research* 8: 118-122.
- SOLHEIM H. & LÅNGSTRÖM B., 1991 — Blue-stain fungi associated with *Tomicus piniperda* in Sweden and preliminary observations on their pathogenicity. *Annales des Sciences Forestières* 48: 149-156.
- SWOFFORD D.L., 1998 — PAUP. Phylogenetic analysis using parsimony (and other methods), Version 4.0 Beta Version. Sunderland, Massachusetts, Sinauer Associates.
- TAMURA K., DUDLEY J., NEI M. & KUMAR S., 2007 — MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* 24: 1596-1599.
- THWAITES J.M., FARRELL R.L., DUNCAN S.M., REAY S.D., BLANCHETTE R.A., HADAR E., HADAR Y., HARRINGTON T.C. & MCNEW D., 2005 — Survey of potential sapstain fungi on *Pinus radiata* in New Zealand. *New Zealand Journal of Botany* 43: 653-663.
- VENN K., 1972 — Discoloration and microflora in stored pulpwood of birch (*Betula pubescens* Ehrh.) in Norway. *Meddelelser fra Det norske Skogforsøksvesen* 15: 219-257.
- VENN K.O., 1986 — Occurrences of *Ceratocystis ulmi* in Norway? *EPPO Bulletin* 16: 513-515.

- VIIRI H., 1997 — Fungal associates of the spruce bark beetle *Ips typographus* L. (Col. Scolytidae) in relation to different trapping methods. *Journal of Applied Entomology* 121: 529-533.
- WHITE T.J., BRUNS T., LEE S. & TAYLOR J., 1990 — Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, (eds), *PCR Protocols: A sequencing guide to methods and applications*. Academic Press, San Diego, pp. 315-322.
- WINGFIELD M.J., SEIFERT K.A. & WEBBER J.F., 1993 — *Ceratocystis* and *Ophiostoma*: Taxonomy, Ecology and Pathogenicity. St. Paul, Minnesota, APS Press.
- ZHOU X.D., DE BEER Z.W., AHUMADA R., WINGFIELD B.D. & WINGFIELD M.J., 2004 — *Ophiostoma* and *Ceratocystiopsis* spp. associated with two pine-infesting bark beetles in Chile. *Fungal Diversity* 15: 261-274.
- ZHOU X.D., DE BEER Z.W. & WINGFIELD M.J., 2006 — DNA sequence comparisons of *Ophiostoma* spp., including *Ophiostoma aurorae* sp. nov., associated with pine bark beetles in South Africa. *Studies in Mycology* 55: 269-277.
- ZIPFEL R.D., DE BEER Z.W., JACOBS K., WINGFIELD M.J. & WINGFIELD B.D., 2006 — Multigene phylogenies define *Ceratocystiopsis* and *Grosmannia* distinct from *Ophiostoma*. *Studies in Mycology* 55: 75-97.